JC05 Rec'd PCT/PTO 30 AUG 2001

FORM PTO-1390 (REV 1-98)	ATTORNEY DOCKET NUMBER 0263-4051
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	U S APPLICATION NO (If known see 37 CFR 1 51) TBA 09/914641

INTERNATIONAL APPLICATION	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/01160	29 February 2000 (29.02.00)	01 March 1999 (01.03.99)

TITLE OF INVENTION
LONG-TERM STABILIZED FORMULATIONS

APPLICANT(S) FOR DO/EO/US
Yasushi SATO

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))

   a. ☒ is transmitted herewith.
   b ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☒ A translation of the International application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (executed)

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included.

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification
15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or Information:
    Copies of the following:
    PCT Request – Four (4) Sheets with verification of translation
    Check in the amount of $1,400.00
    Notification Concerning Submission of Transmittal of Priority Document
    1st page of Published Application W0 00/51629
    Translation of International Preliminary Examination Report (5 pages)
    Notification Informing Applicant of the Communication of the International Application to the Designated Offices.
    Notification Concerning Elected Offices Notified by Their Election.
    Verified certificate of express mail No.: EL762622809US
    Return postcard.

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U.S. APPLICATION NO (if known, see 37 C F R 1 51)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NO
TBA 09/914641	PCT/JP00/01160	0263-4051

17. ☒ The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ... $860.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2) paid to USPTO $710.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33 (1) - (4) $690.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) - (4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of **$130** for furnishing the oath or declaration later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$--	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	30- 20 =	10	X $18.00	$ 180.00	
Independent claims	7 - 3 =	4	X $80.00	$- 320.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00		

TOTAL OF ABOVE CALCULATIONS =	$1,360.00	
Reduction of ½ for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).	$ 0.00	
SUBTOTAL =	$1,360.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$--	
TOTAL NATIONAL FEE =	$1,360.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property =	$ 40.00	
TOTAL FEES ENCLOSED	$1,400.00	
Amount to be refunded:	$	
charged	$	

a. ☒ A check in the amount of $1,400.00 cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 13-4500 in the amount of $0.00 to cover the above fees.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-4500, ORDER NO. 0263-4051 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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Eugene Moroz

Eugene Moroz

Registration Number 25,237

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Docket No. 0263-4051

09/914641

IN THE UNITED STATES

[ ] RECEIVING OFFICE (RO/US)
[X] DESIGNATED OFFICE (DO/US)
[X] ELECTED OFFICE (EO/US)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/01160	29 February 2000 (29.02.00)	01 March 1999 (01.03.99)

TITLE OF INVENTION
LONG-TERM STABILIZED FORMULATIONS

APPLICANT(S)
Yasushi SATO

Commissioner for Patents
Box PCT
Washington, D.C. 20231
Attention: DO/EO/US

VERIFIED CERTIFICATION OF EXPRESS MAILING DATE
(INTERNATIONAL APPLICATION (37 CFR 1.10(c))

I declare that on 30 August 2001 I deposited with the United States Postal Service in an envelope "Express Mail, Post Office to Addressee", bearing Label Number **EL762622809US**, addressed to the "Commissioner for Patents, Box PCT, Washington, D.C. 20231, Attention: DO/EO/US" and having an express mail certification which I executed, the following papers:

International application, consisting of:

PCT Request – Four (4) Sheets with verification of translation
Check in the amount of $1,400.00
Notification Concerning Submission of Transmittal of Priority Document
1st page of Published Application W0 00/51629
Translation of International Preliminary Examination Report (5 pages)
Notification Informing Applicant of the Communication of the International Application to the Designated Offices.
Notification Concerning Elected Offices Notified by Their Election.
Executed Declaration and Power of Attorney
Specification 30 pages, claims 4 pages and 1 page abstract, three sheets of drawings
Transmittal letter to US Designated/Elected Office Concerning Filing under 35USC 371
Assignment & Recordation (3 pages)
Information Disclosure Statement and form 1449 with ten (10) references.
Preliminary Amendment
Return postcard.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

Joan E. Federici
(Typed or printed name of person making this verified statement)

Date August 30, 2001

Joan E. Federici
(Signature of person making this verified statement)

(Verified Certification of Express Mailing Date (International Application) [13-12])

09/914641 PATENT
Docket No. 0263-4051
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	SATO	Group Art Unit	: TBA
Int'l Application	:	PCT/JP00/01160	Examiner	: TBA
Filed	:	29 February 2000		
For	:	**LONG-TERM STABILIZED FORMULATIONS**		

PCT/JP00/01160 29 FEB 2000

PRELIMINARY AMENDMENT

Commissioner Of Patents
Washington, D.C. 20231

Sir:

Please enter the following Preliminary Amendment prior to consideration of the application on the merits and calculation of the filing fee. A copy of the amended claims showing deletions are included in an Appendix to this paper.

IN THE CLAIMS

Please amend Claims 6-9, 12, 15,18, 21, 26, 27, and 30 to read as follows:

6. The G-CSF formulation of Claim 1, which is substantially free from protein as a stabilizer.

7. The G-CSF formulation of Claim 1 in the form of a lyophilized formulation.

8. The G-CSF formulation of Claim 1, further containing mannitol.

9. The G-CSF formulation of Claim 1, further containing a surfactant.

12. The G-CSF formulation of Claim 1, which has a pH of 5-7.

15. The G-CSF formulation of Claim 1 wherein G-CSF is produced from CHO cells.

18. The G-CSF formulation of Claim 15, which has a pH of 6.5.

21. The G-CSF formulation of Claim 19, which is substantially free from protein as a stabilizer.

26. The method of Claim 22 wherein other proteins are not present as stabilizers.

27. The method of Claim 22 wherein said composition containing a physiologically active protein having a methionine residue is lyophilized or in the form of a solution.

30. The composition of Claim 28, which is substantially free from other proteins as stabilizers.

REMARKS

Applicants have amended the claims to eliminate multiple dependent claims to minimize cost associated with filing this national phase application. Specific deletions are shown in an Appendix to this paper. The amendment to the claims does not introduce new matter. Entry of this Preliminary Amendment is respectfully requested.

Respectfully submitted,
MORGAN & FINNEGAN, L.L.P.

Dated: August 30, 2001

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APPENDIX

6. The G-CSF formulation of [any one of] Claim[s] 1 [to 5], which is substantially free from protein as a stabilizer.

7. The G-CSF formulation of [any one of] Claim[s] 1 [to 6] in the form of a lyophilized formulation.

8. The G-CSF formulation of [any one of] Claim[s] 1 [to 7] further containing mannitol.

9. The G-CSF formulation of [any one of] Claim[s] 1 [to 8] further containing a surfactant.

12. The G-CSF formulation of [any one of] Claim[s] 1 [to 11], which has a pH of 5-7.

15. The G-CSF formulation of [any one of] Claim[s] 1 [to 14] wherein G-CSF is produced from CHO cells.

18. The G-CSF formulation of Claim 15 [or 16], which has a pH of 6.5.

21. The G-CSF formulation of Claim 19 [or 20], which is substantially free from protein as a stabilizer.

26. The method of [any one of] Claim[s] 22 [to 25] wherein other proteins are not present as stabilizers.

27. The method of [any one of] Claim[s] 22 [to 26] wherein said composition containing a physiologically active protein having a methionine residue is lyophilized or in the form of a solution.

30. The composition of Claim 28 [or 29], which is substantially free from other proteins as stabilizers.

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SPECIFICATION

LONG-TERM STABILIZED FORMULATIONS

FIELD OF THE INVENTION

The present invention relates to G-CSF (granulocyte colony-stimulating factor) formulations, and particularly stabilized G-CSF formulations showing low active ingredient loss and low content of Met-oxidized G-CSF.

PRIOR ART

G-CSF is a glycoprotein having a molecular weight of about 20,000 and acting on precursor cells of neutrophils to promote their proliferation and differentiation to maturation.

Since we purified high-purity human G-CSF by culturing a cell line collected from tumor cells of a patient with cancer of the floor of the mouth, the human G-CSF gene was successfully cloned and, at present, recombinant human G-CSF can be produced in mass in microorganisms or animal cells by genetic engineering techniques. We also succeeded in converting this purified G-CSF into formulated products supplied to the market as antiinfective agents (Japanese Patent No. 2116515).

G-CSF is used in a very small amount, ie, a formulation containing 0.1-1000 μg (preferably 5-500 μg) of G-CSF is normally administered once to seven times per week per adult. However, this G-CSF is adsorptive to the walls of ampoules, syringes or the like. G-CSF is unstable and susceptible to extrinsic factors such as temperature, humidity, oxygen, UV rays or the like to undergo physical

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or chemical changes including association, polymerization or oxidation, resulting in substantial loss of activity.

Thus, various formulation designs were made to supply stable G-CSF formulations to the market. For example, formulations containing at least one member selected from the group consisting of (a) at least one amino acid selected from threonine, tryptophan, lysine, hydroxylysine, histidine, arginine, cysteine, cystine and methionine; (b) at least one sulfur-containing reducing agent; or (c) at least one antioxidant were proposed (Japanese Patent No. 2577744). G-CSF formulations containing a surfactant such as a Polysorbate as a stabilizer were also proposed (JPA No. 146826/88).

Lyophilized G-CSF formulations containing maltose, raffinose, sucrose, trehalose or an aminosugar were also reported, which are advantageous from the viewpoint of limiting deposits on the container to suppress chemical changes (JPA No. 504784/96).

Some products currently on the market contain a protein commonly used as a stabilizer such as human serum albumin or purified gelatin for suppressing such chemical or physical changes. However, addition of a protein as a stabilizer had problems such as the necessity of a very complicated process for removing contamination with viruses.

However, Met-oxidized G-CSF are more likely to be produced in the absence of such a protein, leading to the problem of deterioration.

DISCLOSURE OF THE INVENTION

An object of the present invention is to provide a G-CSF formulation, which is more stable even during long-term storage and which has a low content of Met-oxidized G-CSF.

As a result of careful studies to achieve the above object, we accomplished the present invention on the basis of the finding that a G-CSF formulation showing a high residual ratio of G-CSF and a low content of Met-oxidized G-CSF even after long-term storage can be obtained by adding a combination of specific amino acids as a stabilizer.

Accordingly, the present invention provides a stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for 2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks and a content of Met-oxidized G-CSF of 1% or less after accelerated testing at 50°C for 1 month or after accelerated testing at 60°C for 2 weeks.

The present invention also provides said G-CSF formulation containing one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, threonine and asparagine; one or more amino acids selected from hydrophobic amino acids; and methionine.

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The present invention also provides said G-CSF formulation wherein said hydrophobic amino acid is selected from phenylalanine, tryptophan and leucine.

The present invention also provides said G-CSF formulation containing one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid and glutamic acid; one or more amino acids selected from the group consisting of phenylalanine, tryptophan and leucine; and methionine.

The present invention also provides said G-CSF formulation containing phenylalanine, arginine and methionine.

The present invention also provides said G-CSF formulation substantially free from protein as a stabilizer.

The present invention also provides said G-CSF formulation in the form of a lyophilized formulation.

The present invention also provides said G-CSF formulation further containing mannitol.

The present invention also provides said G-CSF formulation further containing a surfactant.

The present invention also provides said G-CSF formulation wherein said surfactant is a polyoxyethylene sorbitan alkyl ester.

The present invention also provides said G-CSF formulation wherein said surfactant is Polysorbate 20 and/or 80.

The present invention also provides said G-CSF formulation having a pH of 5-7.

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The present invention also provides said G-CSF formulation having a pH of 5.5-6.8.

The present invention also provides said G-CSF formulation having a pH of 6.5.

The present invention also provides said G-CSF formulation wherein G-CSF is produced from CHO cells.

The present invention also provides a stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for 2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks, characterized in that it contains one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, threonine and asparagine; and one or more amino acids selected from hydrophobic amino acids; and it has a pH of 5-7.

The present invention also provides a stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for 2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks, characterized in that it contains one or more amino acids selected from the

group consisting of lysine, histidine, arginine, aspartic acid and glutamic acid; and one or more amino acids selected from the group consisting of phenylalanine, tryptophan and leucine; and it has a pH of 5-7.

The present invention also provides either one of said G-CSF formulations having a pH of 6.5.

The present invention also provides a method for inhibiting a physiologically active protein containing a methionine residue from producing a variant oxidized at the methionine residue, comprising adding methionine to a composition containing said protein.

The present invention also provides said method wherein said physiologically active protein is a cytokine or a physiologically active peptide.

The present invention also provides said method wherein said physiologically active protein is a colony-stimulating factor or PTH.

The present invention also provides said method wherein said physiologically active protein is G-CSF, erythropoietin or PTH.

The present invention also provides said method wherein other proteins are not present as stabilizers.

The present invention also provides said method wherein said composition containing a physiologically active protein having a methionine residue is lyophilized or in the form of a solution.

The present invention also provides a stabilized composition containing a physiologically active protein

having a methionine residue, further containing methionine and one or more other amino acids.

The present invention also provides said stabilized composition containing a physiologically active protein having a methionine residue wherein said amino acid is one or more selected from the group of consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, phenylalanine, tryptophan, leucine, isoleucine, valine, alanine, proline, glycine, serine, threonine, asparagine, glutamine and tyrosine.

The present invention also provides said stabilized composition containing a physiologically active protein having a methionine residue, characterized in that it is free from other proteins as stabilizers.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows chromatograms of samples 34 and 36 as assayed by method 2 described later after accelerated testing at 60°C for 2 weeks.

FIG. 2 shows chromatograms of samples 34-36 as assayed by method 2 described later immediately after preparation and after accelerated testing at 50°C for 1 month.

FIG. 3 shows HPLC chromatograms of parathyroid hormone solution formulations as assayed by the method shown in example 6 (storage at 50°C for 3 days), which demonstrate that addition of methionine has an inhibitory effect against oxidation of methionine residues. In FIG. 3, the highest peaks at the center correspond to intact PTH,

and peaks designated as Met-8 and Met-18 correspond to PTH oxidized at the 8th methionine residue and the 18th methionine residue, respectively.

THE MOST PREFERRED EMBODIMENTS OF THE INVENTION

G-CSF used for formulations of the present invention includes any high-purity human G-CSF. Specifically, it may be derived from natural sources or obtained by genetic recombination so far as it has substantially the same biological activity as that of mammalian, particularly human G-CSF. Genetically recombinant G-CSF may have the same amino acid sequence as that of natural G-CSF or may contain deletion, substitution or addition of one or more amino acids in said amino acid sequence so far as it has said biological activity. G-CSF in the present invention may be prepared by any process, eg, it may be extracted and purified by various techniques from cultures of a human tumor cell line or may be produced by genetic engineering in cells of E. coli, yeast, Chinese hamster ovary (CHO), C127 or the like and then extracted and purified by various techniques. Preferably, G-CSF is produced in E. coli, yeast or CHO cells by genetic recombination. Most preferably, G-CSF is produced in CHO cells by genetic recombination.

Preferably, G-CSF formulations of the present invention are substantially free from protein such as human serum albumin or purified gelatin as a stabilizer.

G-CSF formulations of the present invention are very stable as compared with previously known G-CSF formulations

as they have a residual ratio of G-CSF of 90% or more, preferably 95% or more after long-term storage testing at 25°C for 3 months, or a residual ratio of G-CSF of 90% or more, preferably 95% or more after long-term storage testing at 40°C for 2 months, or a residual ratio of G-CSF of 90% or more, preferably 95% or more after accelerated testing at 50°C for 1 month, or a residual ratio of G-CSF of 90% or more, preferably 95% or more after accelerated testing at 60°C for 2 weeks and a content of Met-oxidized G-CSF of 1% or less, preferably below detection limit after accelerated testing at 50°C for 1 month or after accelerated testing at 60°C for 2 weeks.

An example of G-CSF formulations of the present invention is a G-CSF formulation containing one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, threonine and asparagine, preferably one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid and glutamic acid; one or more amino acids selected from hydrophobic amino acids, preferably one or more amino acids selected from the group consisting of phenylalanine, tryptophan and leucine; and methionine.

Another example of G-CSF formulations of the present invention is a stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for

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2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks and a content of Met-oxidized G-CSF of 1% or less after accelerated testing at 50°C for 1 month or after accelerated testing at 60°C for 2 weeks, characterized in that it contains one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, threonine and asparagine, preferably one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid and glutamic acid; one or more amino acids selected from hydrophobic amino acids, preferably one or more amino acids selected from the group consisting of phenylalanine, tryptophan and leucine; and methionine; and it has a pH of 5-7.

Amino acids used in the present invention include free amino acids and salts thereof such as sodium salts, potassium salts and hydrochlorides. Formulations of the present invention may contain D-, L- and DL-variants of these amino acids, more preferably L-variants and salts thereof.

The amount of amino acids to be added to formulations of the present invention can be determined in a preferred range using the test method described later depending on the type of the amino acid used. Generally, a final dose of 0.001-50 mg/ml is added. For example, phenylalanine is preferably added at 0.1-25 mg/ml, more preferably 1-20 mg/ml, arginine is preferably added at 0.1-25 mg/ml, more

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preferably 1-20 mg/ml, and methionine is preferably added at 0.001-5 mg/ml, more preferably 0.01-4 mg/ml.

Formulations of the present invention may contain isotonizing agents, eg, polyethylene glycol; and sugars such as dextran, mannitol, sorbitol, inositol, glucose, fructose, lactose, xylose, mannose, maltose, sucrose and raffinose. Mannitol is especially preferred. The amount of mannitol added into formulations is 1-100 mg/ml, more preferably 5-60 mg/ml.

Formulations of the present invention may further contain surfactants. Typical examples of surfactants include:

nonionic surfactants, eg, sorbitan fatty acid esters such as sorbitan monocaprylate, sorbitan monolaurate, sorbitan monopalmitate; glycerin fatty acid esters such as glycerin monocaprylate, glycerin monomyristate, glycerin monostearate; polyglycerin fatty acid esters such as decaglyceryl monostearate, decaglyceryl distearate, decaglyceryl monolinoleate; polyoxyethylene sorbitan fatty acid esters such as polyoxyethylene sorbitan monolaurate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene sorbitan trioleate, polyoxyethylene sorbitan tristearate; polyoxyethylene sorbitol fatty acid esters such as polyoxyethylene sorbitol tetrastearate, polyoxyethylene sorbitol tetraoleate; polyoxyethylene glycerin fatty acid esters such as polyoxyethylene glyceryl monostearate; polyethylene glycol

fatty acid esters such as polyethylene glycol distearate; polyoxyethylene alkyl ethers such as polyoxyethylene lauryl ether; polyoxyethylene polyoxypropylene alkyl ethers such as polyoxyethylene polyoxypropylene glycol ether, polyoxyethylene polyoxypropylene propyl ether, polyoxyethylene polyoxypropylene cetyl ether; polyoxyethylene alkyl phenyl ethers such as polyoxyethylene nonyl phenyl ether; polyoxyethylene hardened castor oils such as polyoxyethylene castor oil, polyoxyethylene hardened castor oil (polyoxyethylene hydrogenated castor oil); polyoxyethylene beeswax derivatives such as polyoxyethylene sorbitol beeswax; polyoxyethylene lanolin derivatives such as polyoxyethylene lanolin; polyoxyethylene fatty acid amides such as polyoxyethylene stearic acid amide having an HLB of 6-18;

anionic surfactants, eg, alkyl sulfates having a C10-18 alkyl group such as sodium cetylsulfate, sodium laurylsulfate, sodium oleylsulfate; polyoxyethylene alkyl ether sulfates having an average EO mole number of 2-4 and a C10-18 alkyl group such as sodium polyoxyethylene laurylsulfate; alkyl sulfosuccinic acid ester salts having a C8-18 alkyl group such as sodium laurylsulfosuccinate; and

natural surfactants, eg, lecithin; glycerophospholipids; sphingophospholipids such as sphingomyelin; sucrose fatty acid esters of C12-18 fatty acids. One or more of these surfactants may be added in combination to formulations of the present invention.

Preferred surfactants are polyoxyethylene sorbitan fatty acid esters, more preferably Polysorbates 20, 21, 40, 60, 65, 80, 81, 85, most preferably Polysorbates 20 and 80.

The amount of surfactants to be added to G-CSF-containing formulations of the present invention is typically 0.0001-1 parts by weight per part by weight of G-CSF, preferably 0.01-5 parts by weight per part by weight of G-CSF, and most preferably 0.2-2 parts by weight per part by weight of G-CSF.

Preferably, G-CSF formulations of the present invention have a pH of 5-7, more preferably 5.5-6.8, still more preferably 6-6.7, and most preferably 6.5.

G-CSF formulations of the present invention may further contain diluents, solubilizing agents, excipients, pH-modifiers, soothing agents, buffers, sulfur-containing reducing agents, antioxidants or the like, if desired. For example, sulfur-containing reducing agents include N-acetylcysteine, N-acetylhomocysteine, thioctic acid, thiodiglycol, thioethanolamine, thioglycerol, thiosorbitol, thioglycolic acid and salts thereof, sodium thiosulfate, glutathione, and sulfhydryl-containing compounds such as thioalkanoic acid having 1 to 7 carbon atoms. Antioxidants include erythorbic acid, dibutylhydroxytoluene, butylhydroxyanisole, α-tocopherol, tocopherol acetate, L-ascorbic acid and salts thereof, L-ascorbyl palmitate, L-ascorbyl stearate, sodium bisulfite, sodium sulfite, triamyl gallate, propyl gallate or chelating agents such as disodium ethylenediamine tetraacetate (EDTA), sodium

pyrophosphate, sodium metaphosphate. Other components commonly added may also be contained, eg, inorganic salts such as sodium chloride, potassium chloride, calcium chloride, sodium phosphate, potassium phosphate, sodium bicarbonate; and organic salts such as sodium citrate, potassium citrate, sodium acetate.

G-CSF formulations of the present invention include solution formulations, lyophilized formulations, spray-dried formulations, etc. Lyophilized formulations are most preferred.

Formulations of the present invention can be prepared by dissolving these components in an aqueous buffer known in the art of solution formulations such as phosphate buffers (preferably sodium monohydrogen phosphate - sodium dihydrogen phosphate system) and/or citrate buffers (preferably sodium citrate buffer) to prepare a solution formulation, or lyophilizing or spray-drying thus prepared solution formulation by standard procedures.

Stabilized G-CSF-containing formulations of the present invention are normally administered via parenteral routes such as injection (subcutaneous, intravenous or intramuscular injection) or percutaneous, mucosal, nasal or pulmonary administration, but may also be orally administered.

G-CSF formulations of the present invention are normally packed in a sealed and sterilized plastic or glass container, and dissolved in pure water (sterilized water for injection) before use.

The amount of G-CSF contained in formulations of the present invention can be determined depending on the type of the disease to be treated, the severity of the disease, the age of the patient or other factors, but generally ranges from 1 to 1000 μg/mL, preferably 10 to 800 μg/mL, more preferably 50 to 500 μg/mL.

Formulations of the present invention are clinically very useful as they were found to improve protective functions based on immune response such as resistance of the patient or activity when they were coadministered with drugs such as antibiotics, antibacterial agents or anticancer agents in the chemotherapy of infectious diseases or cancer. Therefore, formulations of the present invention can be administered in combination with these drugs.

As demonstrated in the examples below, G-CSF formulations of the present invention show a very good residual ratio of G-CSF after long-term storage testing at 25°C for 3 months or long-term storage testing at 40°C for 2 months or accelerated testing at 50°C for 1 month or accelerated testing at 60°C for 2 weeks. Moreover, a small content of Met-oxidized G-CSF was observed after accelerated testing at 50°C for 1 month or accelerated testing at 60°C for 2 weeks. G-CSF formulations of the present invention have a residual ratio of G-CSF of 90% or more, preferably 95% or more after long-term storage testing at 25°C for 3 months, or a residual ratio of G-CSF of 90% or more, preferably 95% or more after long-term

storage testing at 40°C for 2 months, or a residual ratio of G-CSF of 90% or more, preferably 95% or more after accelerated testing at 50°C for 1 month, or a residual ratio of G-CSF of 90% or more, preferably 95% or more after accelerated testing at 60°C for 2 weeks and a content of Met-oxidized G-CSF of 1% or less, preferably below detection limit after accelerated testing at 50°C for 1 month or after accelerated testing at 60°C for 2 weeks.

It was observed from the results of the examples below that the residual ratio of G-CSF in formulations of the present invention after long-term storage at normal temperatures can be improved by adding one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, threonine and asparagine and one or more amino acids selected from hydrophobic amino acids and that the content of Met-oxidized G-CSF can be kept below detection limit by adding methionine. Without wishing to be bound to any specific theory, we assumed that the added methionine is oxidized in place of methionine residues of G-CSF so that the content of Met-oxidized G-CSF decreases.

When methionine is added to a compositions of a physiologically active protein having a methionine residue, which is more susceptible to production of a variant oxidized at the methionine residue and has a physiological activity in a small amount, said physiologically active protein can also be prevented from producing a variant oxidized at the methionine residue according to the present

invention. Addition of methionine seems to be especially effective when said physiologically active protein composition is free from other proteins as stabilizers or when said protein composition is lyophilized or in the form of a solution because the variant of said protein oxidized at the methionine residue is more likely to be produced in such cases.

When other one or more amino acids are added to a composition of the present invention, a stabilized composition containing a physiologically active protein having a methionine residue can also be prepared, which is inhibited from producing a variant oxidized at the methionine residue as well as inhibited from decomposition, aggregation or the like of said physiologically active protein.

Amino acids that can be added for this purpose include lysine, histidine, arginine, aspartic acid, glutamic acid, phenylalanine, tryptophan, leucine, isoleucine, valine, alanine, proline, glycine, serine, threonine, asparagine, glutamine and tyrosine, preferably histidine, arginine and phenylalanine.

Physiologically active proteins of the present invention include, for example:

cytokines such as interleukins (eg, IL-1 to IL-13), colony-stimulating factors (eg, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO)), interferons (eg,

IFN-α, β, γ), tumor necrosis factors (eg, TNF-α, TNF-β), transforming growth factor (TGF), platelet-derived growth factor (PDGF), LIF (leukemia inhibitory factor), oncostatin M (OSM), migration inhibitory factor (MIF), chemokines (eg, IL-8, LD78, MCP-1);

physiologically active peptides such as insulin, glucagon, parathyroid hormone (PTH), gastrin, selectin, cholecystokinin, gastric inhibitory polypeptides, substance P, motilin, spleen polypeptides, neurotensin, enteroglucagon, gastrin-releasing peptides, somatostatin-28, dynorphin, galanin, vanilone, pancreostatin and zeopsin;

bioenzymes such as enzymes having a methionine residue at the active center (eg, malate dehydrogenase);

or variants thereof.

Physiologically active proteins of the present invention are preferably cytokines or physiologically active peptides, more preferably colony-stimulating factors such as G-CSF or erythropoietin or PTH, still more preferably G-CSF, erythropoietin or PTH.

The following examples further illustrate the present invention, without limiting the same thereto. Various changes and modifications can be made by those skilled in the art on the basis of the description of the invention, and such changes and modifications are also included in the present invention.

EXAMPLES

Test method

Formulated solutions containing various components in

the amounts shown in Tables 1 and 2 below were prepared and aseptic-filtered, and then precisely 1 mL each was aseptically packed in a vial and lyophilized. After completion of lyophilization, the vial was completely capped to prepare G-CSF lyophilized formulations.

Table 1

	G-CSF	Phenylalanine	Arginine	Methionine	Mannitol	Polysorbate 20	pH buffer
Sample 1	250 µg	10 mg	10 mg	0	50 mg	0.1 mg	Phosphate, pH 7.4
Sample 2	250 µg	10 mg	10 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5

	G-CSF	Phenylalanine	Arginine	Methionine	Mannitol	Polysorbate 20	pH buffer
Sample 3	100 µg	0 mg	0 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 4	100 µg	10 mg	0 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 5	100 µg	0 mg	10 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 6	100 µg	10 mg	10 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5

	G-CSF	Phenylalanine	Arginine	Methionine	Mannitol	Polysorbate 20	pH buffer
Sample 7	250 µg	0 mg	0 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 8	250 µg	10 mg	0 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 9	250 µg	0 mg	10 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 10	250 µg	10 mg	10 mg	0	50 mg	0.1 mg	Phosphate, pH 6.5

	G-CSF	Amino acid 1	Amino acid 2	Methionine	Mannitol	Polysorbate 20	pH buffer
Sample 11	100 µg	Phenylalanine	Lysine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 12	100 µg	Phenylalanine	Histidine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 13	100 µg	Phenylalanine	Arginine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 14	100 µg	Phenylalanine	Aspartic acid	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 15	100 µg	Phenylalanine	Glutamic acid	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 16	100 µg	Phenylalanine	Serine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 17	100 µg	Phenylalanine	Threonine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 18	100 µg	Phenylalanine	Tyrosine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 19	100 µg	Phenylalanine	Asparagine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 20	100 µg	Phenylalanine	Glutamine	0	50 mg	0.1 mg	Phosphate, pH 6.5

In each case, 10 mg phenylalanine was added (equivalent to 60 mM). Amino acid 2 was added in an amount equivalent to 60 mM (equimolar to amino acid 1).

	G-CSF	Amino acid 1	Amino acid 2	Methionine	Mannitol	Polysorbate 20	pH buffer
Sample 21	100 μg	Arginine	Alanine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 22	100 μg	Arginine	Valine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 23	100 μg	Arginine	Leucine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 24	100 μg	Arginine	Isoleucine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 25	100 μg	Arginine	Methionine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 26	100 μg	Arginine	Tryptophan	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 27	100 μg	Arginine	Phenylalanine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 28	100 μg	Arginine	Proline	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 29	100 μg	Arginine	Glycine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 30	100 μg	Arginine	Serine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 31	100 μg	Arginine	Threonine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 32	100 μg	Arginine	Asparagine	0	50 mg	0.1 mg	Phosphate, pH 6.5
Sample 33	100 μg	Arginine	Glutamine	0	50 mg	0.1 mg	Phosphate, pH 6.5

In each case, 10 mg arginine was added (equivalent to 60 mM). Amino acid 2 was added in an amount equivalent to 60 mM (equimolar to amino acid 1).

Table 2

	G-CSF	Phe	Arg	Met	Mannitol	Polysorbate 20	pH buffer
Sample 34	100 μg	10 mg	10 mg	0 mg	25 mg	0.1 mg	Phosphate, pH 6.5
Sample 35	100 μg	10 mg	10 mg	0.1 mg	25 mg	0.1 mg	Phosphate, pH 6.5
Sample 36	100 μg	10 mg	10 mg	1 mg	25 mg	0.1 mg	Phosphate, pH 6.5

Thus aseptically prepared G-CSF-containing lyophilized formulations were allowed to stand in an incubator at 60°C for 2 weeks and 1 month; at 50°C for 1, 2 and 3 months; at 40°C for 2, 4 and 6 months; and at 25°C for 3 and 6 months.

Accelerated and unaccelerated formulations were dissolved in precisely 1 mL of pure water to prepare test samples for the assays described below.

The residual ratio of G-CSF (% residual) in each vial was determined by method 1 below. The content of Met-oxidized G-CSF in each vial was also determined by method 2 below.

Method 1

The content of G-CSF in each sample was assayed by reverse phase high-speed liquid chromatography using a C4 reverse phase column (4.6 mm x 250 mm, 300 angstroms) with a mobile phase consisting of pure water, acetonitrile and trifluoroacetic acid. The amount equivalent to 5 μg of G-CSF was injected and G-CSF was eluted with an acetonitrile gradient and spectroscopically detected at a wavelength of 215 nm.

The G-CSF content determined by this method was used to calculate the percentage (%) of residual G-CSF according to the following equation after acceleration at 60°C for 2 weeks and 50°C for 1 month and after storage at 60°C for 2 weeks and 1 month; 50°C for 1, 2 and 3 months; 40°C for 2, 4 and 6 months; and 25°C for 3 and 6 months.

$$\text{Residual ratio (\%)} = \frac{\text{(G-CSF content after acceleration for a test period)}}{\text{(G-CSF content before acceleration)}} \times 100$$

Method 2

Intact G-CSF and Met-oxidized G-CSF in each sample were assayed by reverse phase high-speed liquid chromatography using a C4 reverse phase column (4.6 mm x 250 mm, 300 angstroms) with a mobile phase consisting of

pure water, acetonitrile and trifluoroacetic acid. G-CSF was eluted with an acetonitrile gradient and spectroscopically detected at a wavelength of 215 nm.

The peak areas of intact G-CSF and Met-oxidized G-CSF determined by this method were used to calculate the content of Met-oxidized G-CSF according to the following equation after acceleration at 60°C for 2 weeks and 50°C for 1 month.

$$\text{Content of Met-oxidized G-CSF (\%)} = \frac{\text{(Met-oxidized G-CSF)}}{\text{(intact G-CSF)+(Met-oxidized G-CSF)}} \times 100$$

Example 1: Effect of varying pHs on the residual ratio of G-CSF

The residual ratio of G-CSF in samples 1 and 2 prepared at different pHs shown in Table 1 were calculated according to the equation shown in method 1 after accelerated testing at 60°C for 2 weeks and 50°C for 1 month. The results are shown in Table 3.

Table 3

	Sample 1, pH7.4	Sample 2, pH6.5
50°C, 1 month	97.7	99.7
60°C, 2 weeks	95.8	97.1

Comparable or more stability was observed with the formulation at pH 6.5 as compared with pH 7.4.

Example 2: Effect of various amino acids on the residual ratio of G-CSF (1)

The residual ratio of G-CSF in samples 3-6 (G-CSF content 100 μg) and samples 7-10 (G-CSF content 250 μg) prepared with various amino acids shown in Table 1 were calculated according to the equation shown in method 1 after accelerated testing at 60°C for 2 weeks and 50°C for 1 month. The results are shown in Tables 4 and 5.

Table 4

Formulations containing 100 μg G-CSF

	Sample 3	Sample 4	Sample 5	Sample 6
Phenylalanine	No	10mg	No	10mg
Arginine	No	No	10mg	10mg
50°C, 1 month	72.9%	84.8%	82.4%	98.3%
60°C, 2 weeks	67.2%	77.9%	68.8%	95.0%

Table 5

Formulations containing 250 μg G-CSF

	Sample 7	Sample 8	Sample 9	Sample 10
Phenylalanine	No	10mg	No	10mg
Arginine	No	No	10mg	10mg
50°C, 1 month	76.6%	88.1%	96.3%	99.7%
60°C, 2 weeks	74.0%	78.1%	90.7%	97.1%

At any G-CSF content, stability is improved in formulations added with phenylalanine alone or arginine alone as compared with formulations containing no amino

acid, but to an insufficient extent. Marked improvement in stability was observed by combining phenylalanine with arginine.

Example 3: Effect of various amino acids on the residual ratio of G-CSF (2)

The residual ratio of remaining G-CSF in samples 11-20 (containing phenylalanine as amino acid 1 and any of lysine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, asparagine and glutamine as amino acid 2) and samples 21-33 (containing arginine as amino acid 1 and any of alanine, valine, leucine, isoleucine, methionine, tryptophan, phenylalanine, proline, glycine, serine, threonine, asparagine and glutamine as amino acid 2) prepared with various amino acids shown in Table 1 were calculated according to the equation shown in method 1 after accelerated testing at 60°C for 2 weeks and 50°C for 1 month. The results are shown in Tables 6 and 7.

Table 6

	50°C, 1 month	60°C, 2 weeks
Sample 11	92.8%	91.2%
Sample 12	98.8%	97.5%
Sample 13	98.0%	96.0%
Sample 14	95.7%	96.7%
Sample 15	95.6%	94.0%
Sample 16	88.4%	87.8%
Sample 17	96.4%	90.7%
Sample 18	84.6%	81.7%
Sample 19	95.0%	95.3%
Sample 20	89.8%	87.2%

Table 7

	50°C, 1 month	60°C, 2 weeks
Sample 21	89.0%	84.4%
Sample 22	88.9%	86.5%
Sample 23	96.3%	96.2%
Sample 24	88.5%	89.3%
Sample 25	95.5%	88.5%
Sample 26	101.4%	98.6%
Sample 27	97.0%	95.7%
Sample 28	89.4%	82.5%
Sample 29	90.9%	71.2%
Sample 30	89.2%	85.2%
Sample 31	90.6%	87.3%
Sample 32	94.0%	88.6%
Sample 33	90.1%	84.6%

Marked improvement in long-term storage stability was observed by combining phenylalanine with lysine,

phenylalanine with histidine, phenylalanine with arginine, phenylalanine with aspartic acid, phenylalanine with glutamic acid, phenylalanine with threonine, or phenylalanine with asparagine, or combining arginine with leucine, arginine with tryptophan, or arginine with phenylalanine.

Example 4: Long-term storage testing

The residual ratio of G-CSF in samples containing 100 μg or 250 μg G-CSF and 10 mg phenylalanine, 10 mg arginine and 1 mg methionine were calculated according to the equation shown in method 1 after storage at 60°C for 2 weeks and 1 month; 50°C for 1, 2 and 3 months; 40°C for 2, 4 and 6 months; and 25°C for 3 and 6 months. The results are shown in Table 8.

Table 8

G-CSF (μg)	60°C		50°C			40°C			25°C	
	2W[1]	1M[2]	1M	2M	3M	2M	4M	6M	3M	6M
100	98.3	96.2	99.9	100.1	95.9	101.0	100.0	98.8	97.0	98.0
250	97.2	94.5	98.7	98.0	96.7	99.4	99.3	98.1	98.5	100.6

1: week

2: month

Both formulations showed excellent residual ratio of G-CSF.

Example 5: Effect of addition of amino acids on the content of Met-oxidized G-CSF.

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An example of chromatograms of samples 34-36 prepared with methionine in various amounts shown in Table 2 (containing a fixed amount of phenylalanine and arginine and 0 mg, 0.1 mg or 1 mg methionine) as assayed by method 2 after accelerated testing at 60°C for 2 weeks is shown in Fig. 1 and an example of chromatograms of said samples as assayed by method 2 immediately after preparation and after accelerated testing at 50°C for 1 month is shown in Fig. 2.

Content Met-oxidized G-CSF was observed both immediately after preparation and after storage at 50°C for 1 month of the sample containing no methionine (sample 34), while content of Met-oxidized G-CSF could be completely inhibited even after long-term storage by adding 0.1 mg or more of methionine.

The results of the content of Met-oxidized G-CSF calculated by the equation shown in method 2 are shown in table 9.

Table 9

	Sample 34 0mg Met	Sample 35 0.1mg Met	Sample 36 1mg Met
50°C, 1 month	1.2%	N.D.	N.D.
60°C, 2 weeks	1.7%	N.D.	N.D.

N.D.: below detection limit.

Thus, content of Met-oxidized G-CSF could be completely inhibited by adding 0.1 mg or more of methionine.

Example 6: Addition of methionine to parathyroid hormone

solution formulations has an inhibitory effect against oxidation of methionine residues

Formulated solutions of samples 37-39 containing 200 µg/mL of parathyroid hormone having 1-84 residues (hereinafter abbreviated as PTH) (prepared by the process described in WO9014415) and other components in the amounts shown in Table 10 below were prepared and aseptic-filtered, and then precisely 1 mL each was aseptically packed in a vial, which was completely capped to prepare PTH solution formulations.

Table 10

	PTH	Methionine	Polysorbate 20	pH (Citrate/phosphate buffer)
Sample 37	200 µg/mL	No	0.01%	6.5
Sample 38	200 µg/mL	0.01%	0.01%	6.5
Sample 39	200 µg/mL	0.1%	0.01%	6.5

Thus aseptically prepared PTH-containing solution formulations were allowed to stand in an incubator at 50°C for 3 days.

The PTH content in each sample was assayed by reverse phase high-speed liquid chromatography using a C18 reverse phase column (4.6 mm x 250 mm, 300 angstroms) with a mobile phase consisting of pure water, acetonitrile and trifluoroacetic acid. The amount equivalent to 10 µg of PTH was injected and PTH was eluted with an acetonitrile gradient and spectroscopically detected at a wavelength of 215 nm.

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In this test series, PTH oxidized at the 8th methionine residue and PTH oxidized at the 18th methionine residue are detected immediately before intact PTH as shown in Fig. 3. The chromatograms show that oxidation at the 8th methionine residue in PTH and oxidation at the 18th methionine residue in PTH can be inhibited by adding methionine to formulations. They also show that addition of methionine into formulations has no influence on chemical decomposition reactions other than those of methionine residues. That is, addition of methionine into formulations can specifically improve only inhibition of oxidation of the protein at methionine residues without influencing other chemical decomposition reactions.

INDUSTRIAL APPLICABILITY

G-CSF formulations of the present invention are stable formulations that show a very high residual ratio of G-CSF even after long-term storage and that can almost completely inhibit oxidation of G-CSF at methionine residues.

CLAIMS

1. A stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for 2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks and a content of Met-oxidized G-CSF of 1% or less after accelerated testing at 50°C for 1 month or after accelerated testing at 60°C for 2 weeks.

2. The G-CSF formulation of Claim 1 containing one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, threonine and asparagine; one or more amino acids selected from hydrophobic amino acids; and methionine.

3. The G-CSF formulation of Claim 2 wherein said hydrophobic amino acid is selected from phenylalanine, tryptophan and leucine.

4. The G-CSF formulation of Claim 1 containing one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid and glutamic acid; one or more amino acids selected from the group consisting of phenylalanine, tryptophan and leucine; and methionine.

5. The G-CSF formulation of Claim 1 containing phenylalanine, arginine and methionine.

6. The G-CSF formulation of any one of Claims 1 to 5,

which is substantially free from protein as a stabilizer.

7. The G-CSF formulation of any one of Claims 1 to 6 in the form of a lyophilized formulation.

8. The G-CSF formulation of any one of Claims 1 to 7 further containing mannitol.

9. The G-CSF formulation of any one of Claims 1 to 8 further containing a surfactant.

10. The G-CSF formulation of Claim 9 wherein said surfactant is a polyoxyethylene sorbitan alkyl ester.

11. The G-CSF formulation of Claim 10 wherein said surfactant is Polysorbate 20 and/or 80.

12. The G-CSF formulation of any one of Claims 1 to 11, which has a pH of 5-7.

13. The G-CSF formulation of Claim 12, which has a pH of 5.5-6.8.

14. The G-CSF formulation of Claim 13, which has a pH of 6.5.

15. The G-CSF formulation of any one of Claims 1 to 14 wherein G-CSF is produced from CHO cells.

16. A stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for 2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks, characterized in that it contains one or more amino acids selected from the group consisting of lysine,

histidine, arginine, aspartic acid, glutamic acid, threonine and asparagine; and one or more amino acids selected from hydrophobic amino acids; and it has a pH of 5-7.

17. A stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for 2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks, characterized in that it contains one or more amino acids selected from the group consisting of lysine, histidine, arginine, aspartic acid and glutamic acid; and one or more amino acids selected from the group consisting of phenylalanine, tryptophan and leucine; and it has a pH of 5-7.

18. The G-CSF formulation of Claim 15 or 16, which has a pH of 6.5.

19. A stabilized G-CSF formulation, which does not substantially produce a variant oxidized at methionine.

20. A stabilized G-CSF formulation, which contains methionine and other one or more amino acids and does not substantially produce a variant oxidized at methionine.

21. The G-CSF formulation of Claim 19 or 20, which is substantially free from protein as a stabilizer.

22. A method for inhibiting a physiologically active protein containing a methionine residue from producing a

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variant oxidized at the methionine residue, comprising adding methionine to a composition containing said protein.

23. The method of Claim 22 wherein said physiologically active protein is a cytokine or a physiologically active peptide.

24. The method of Claim 22 wherein said physiologically active protein is a colony-stimulating factor or PTH.

25. The method of Claim 22 wherein said physiologically active protein is G-CSF, erythropoietin or PTH.

26. The method of any one of Claims 22 to 25 wherein other proteins are not present as stabilizers.

27. The method of any one of Claims 22 to 26 wherein said composition containing a physiologically active protein having a methionine residue is lyophilized or in the form of a solution.

28. A stabilized composition containing a physiologically active protein having a methionine residue, further containing methionine and one or more other amino acids.

29. The composition of Claim 28 wherein said amino acid is one or more selected from the group of consisting of lysine, histidine, arginine, aspartic acid, glutamic acid, phenylalanine, tryptophan, leucine, isoleucine, valine, alanine, proline, glycine, serine, threonine, asparagine, glutamine and tyrosine.

30. The composition of Claim 28 or 29, which is substantially free from other proteins as stabilizers.

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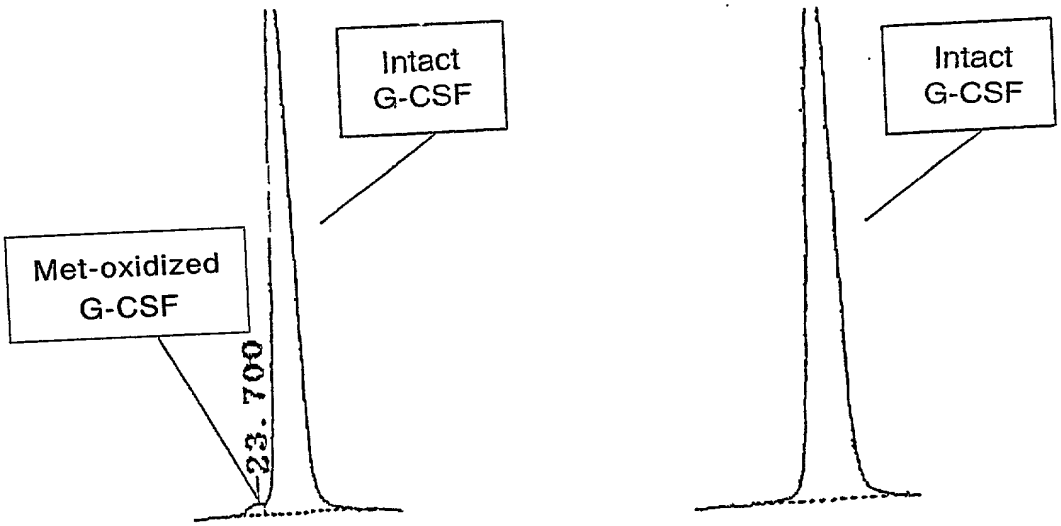
ABSTRACT

A stable G-CSF formulation having a residual ratio of G-CSF of 90% or more after long-term storage testing at 25°C for 3 months or a residual ratio of G-CSF of 90% or more after long-term storage testing at 40°C for 2 months or a residual ratio of G-CSF of 90% or more after accelerated testing at 50°C for 1 month or a residual ratio of G-CSF of 90% or more after accelerated testing at 60°C for 2 weeks and a content of Met-oxidized G-CSF of 1% or less after accelerated testing at 50°C for 1 month or after accelerated testing at 60°C for 2 weeks.

FIG. 1

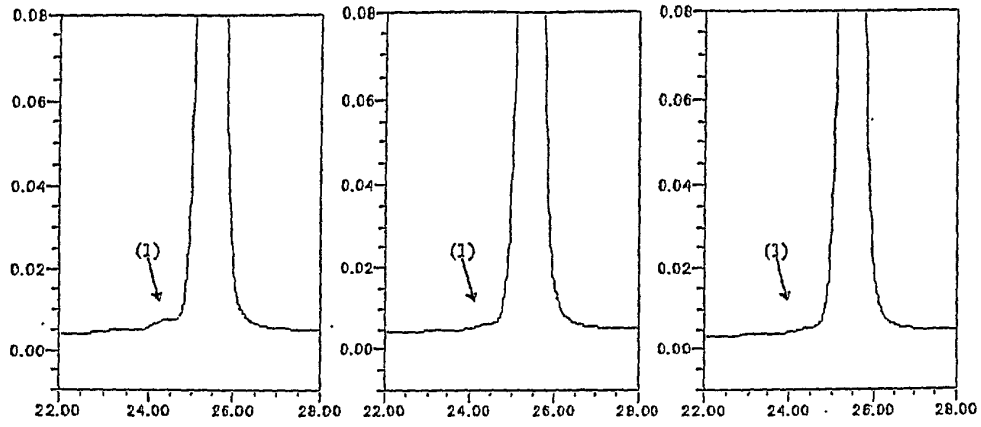


Sample 34

Sample 36

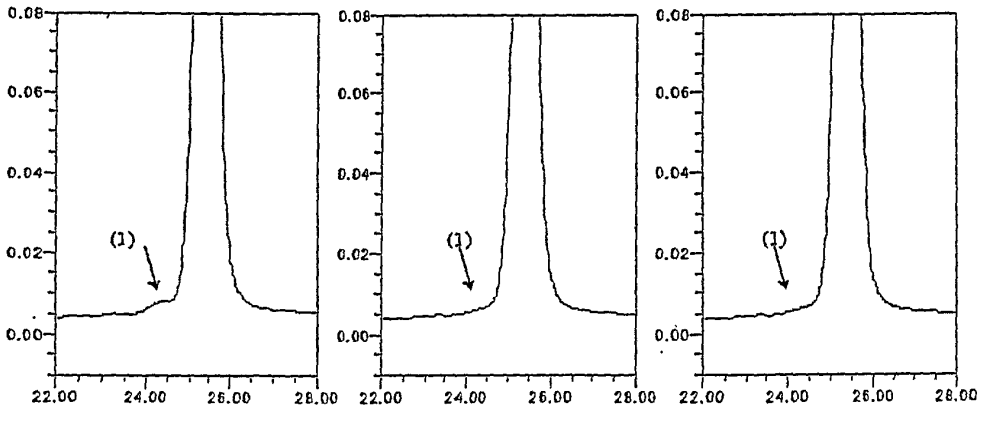
FIG. 2

Before acceleration



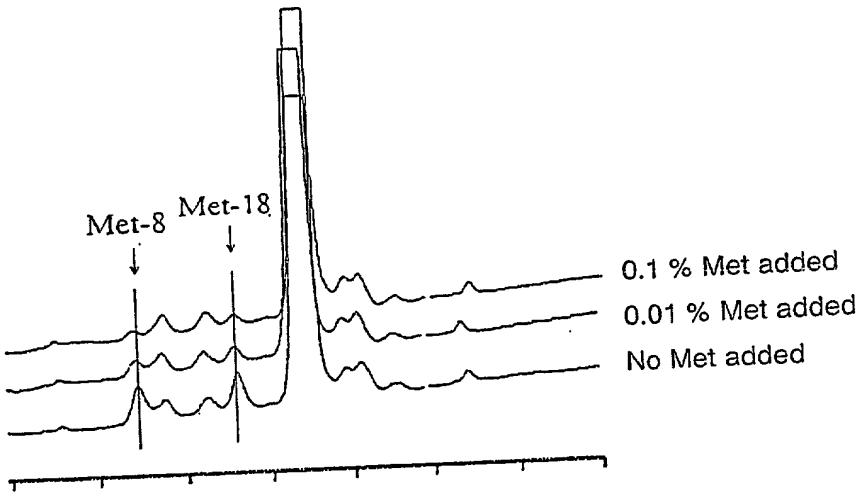
Methionine 0 mg 0.1mg Methionine 1 mg Methionine

After acceleration at 50°C for 1 month



Methionine 0 mg 0.1 mg Methionine 1 mg Methionine

FIG. 3



Docket No. _____

COMBINED DECLARATION AND POWER OF ATTORNEY FOR
ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL
DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART APPLICATION

As a below name inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

LONG-TERM STABILIZED FORMULATIONS

the specification of which

a. [ ] is attached hereto

b. [ ] was filed on _____ as application Serial No. _____ and was amended on _____. (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STATE

c. [X] was described and claimed in International Application No PCT/JP00/01160 filed on Feb. 29, 2000 and as amended on _____. (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby specify the following as the correspondence address to which all communications about this application are to be directed:

SEND CORRESPONDENCE TO: MORGAN & FINNEGAN, L.L.P
345 Park Avenue
New York, N.Y. 10154

DIRECT TELEPHONE CALLS TO: _____
(212) 758-4800

[ ] I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or under § 365(b) of any foreign application(s) for patent or inventor's certificate or under § 365(a) of any PCT international application(s) designating at least one country other than the U.S. listed below and also have identified below such foreign application(s) for patent or inventor's certificate or such PCT international application(s) filed by me on the same subject matter having a filing date within twelve (12) months before that of the application on which priority is claimed:

[x] The attached 35 U.S.C. § 119 claim for priority for the application(s) listed below forms a part of this declaration.

Country/PCT	Application Number	Date of filing (day, month, yr)	Date of Issue (day, month, yr)	Priority Claimed
Japan	52314/1999	1/3/1999		[x] YES [ ] NO
				[ ] YES [ ] NO
				[ ] YES [ ] NO

[ ] I hereby claim the benefit under 35 U.S.C. § 119(e) of any U.S. provisional application(s) listed below.

Provisional Application No.	Date of Filing (day, month, yr)

ADDITIONAL STATEMENTS FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART OR PCT INTERNATIONAL APPLICATION(S) (DESIGNATING THE U.S.)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or under § 365(c) of any PCT international application(s) designating the U.S. listed below.

US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)
US/PCT Application Serial No.	Filing Date	Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)

[ ] In this continuation-in-part application, insofar as the subject matter of any of the claims of this application is not disclosed in the above listed prior United States or PCT international application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or Imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and/or agents with full power of substitution and revocation, to prosecute this application, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith: John A. Diaz (Reg. No. 19,550), John C. Vassil (Reg. No. 19,098), Alfred P. Ewert (Reg. No. 19,887), David H. Pfeffer, P.C. (Reg. No. 19,825), Harry C. Marcus (Reg. No. 22,390), Robert E. Paulson (Reg. No. 21,046), Stephen R. Smith (Reg. No. 22,615), Kurt E. Richter (Reg. No. 24,052), J. Robert Dailey (Reg. No. 27,434), Eugene Moroz (Reg. No. 25,237), John F. Sweeney (Reg. No. 27,471), Arnold I. Rady (Reg. No. 26,601), Christopher A. Hughes (Reg. No. 26,914), William S. Feiler (Reg. No. 26,728), Joseph A.

Calvaruso (Reg. No. 28,287), James W. Gould (Reg. No. 28,859), Richard C. Komson (Reg. No. 27,913), Israel Blum (Reg. No. 26,710), Bartholomew Verdirame (Reg. No. 28,483), Maria C.H. Lin (reg. No. 29,323), Joseph A. DeGirolamo (Reg. No. 28,595), Michael A. Nicodema (Reg. No. 33,199), Michael P. Dougherty (Reg. No. 32,730), Seth J. Atlas (Reg. No. 32,454), Andrew M. Riddles (Reg. No. 31,657), Bruce D. DeRenzi (Reg. No. 33,676), Michael M. Murray (Reg. No. 32,537) and Mark J. Abate (Reg. No. 32,527) of Morgan & Finnegan, L.L.P. whose address is: 345 Park Avenue, New York, New York, 10154; and Edward A. Pennington (Reg. No. 32,588) of Morgan & Finnegan, L.L.P., whose address is 1775 Eye Street, Suite 400, Washington, D.C. 20006.

[x] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from YUASA AND HARA as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

Full name of sole ~~first~~ inventor Yasushi SATO

Inventor's signature* Yasushi Sato date August 13, 2001

Residence Tokyo, Japan JPX

Citizenship Japanese

Post Office Address c/o Chugai Seiyaku Kabushiki Kaisha of 41-8, Takada 3-chome, Toshima-ku, Tokyo 171-8545 Japan

Full name of second joint inventor, if any

Inventor's signature* date

Residence

Citizenship

Post Office Address

[ ] ATTACHED IS ADDED PAGE TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR SIGNATURE BY THIRD AND SUBSEQUENT INVENTORS FORM.

* Before signing this declaration, each person signing must:

1. Review the declaration and verify the correctness of all information therein; and

2. Review the specification and the claims, including any amendments made to the claims.

After the declaration is signed, the specification and claims are not to be altered.

To the inventor(s):

The following are cited in or pertinent to the declaration attached to the accompanying application:

Title 37, Code of Federal Regulation, § 1.56

Duty to disclose information material to patentability.

(a) A patent by its very nature is affect with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

Title 35, U.S. Code § 101

Inventions patentable

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Title 35 U.S. Code § 102

Conditions for patentability; novelty and loss of right to patent

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent,

(b) the invention was patented or described in a printed publication in this or foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States, or

(c) he has abandoned the invention, or

(d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate field more than twelve months before the filing of the application in the United States, or

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or

(f) he did not himself invent the subject matter sought to be patented, or

(g) before the applicant's invention thereof the invention was made in this country by another had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other ...

Title 35, U.S. Code § 103

Conditions for patentability; non-obvious subject matter

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

Title 35, U.S. Code § 112 (in part)

Specification

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms also enable any person skilled in the art to which it pertains, or with which it is mostly nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Title 35, U.S. Code § 119

Benefit of earlier filing date in foreign country; right of priority

An application for patent for an invention filed in this country by any person who has, or whose legal representatives or assigns have, previously regularly filed an application for a patent for the same invention in a foreign country which affords similar privileges in the case of applications filed in the United States or to citizens of the United States, shall have the same effect as the same application would have if filed in this country on the date on which the application for patent for the same invention was first filed in such foreign country, if the application in this country is filed within twelve months from the earliest date on which such foreign application was filed; but no patent shall be granted on any application for patent for an invention which had been patented or described in a printed publication in any country more than one year before the date of he actual filing of the application in this country, or which had been in public use or on sale in this country more than one year prior to such filing.

Title 35, U.S. Code § 120

Benefit or earlier filing date in the United States

An application for patent for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States, or as provided by section 363 of this title, which is filed by an inventor or inventors named in the previously filed application shall have the same effect, as to such invention, as though filed on the date of the prior application, if filed before the patenting or abandonment of or termination of proceedings on the first application or an application similarly entitled to the benefit of the filing date of the first application and if it contains or is amended to contain a specific reference to the earlier filed application.

Please read carefully before signing the Declaration attached to the accompanying Application.

If you have any questions, please contact Morgan & Finnegan, L.L.P.

FORM:COMB-DEC.NY
Rev. 5/21/98

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